

Role of calreticulin in regulating intracellular Ca^{2+} storage and capacitative Ca^{2+} entry in HeLa cells

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Summary Calreticulin is a Ca^{2+} binding protein located primarily in the endoplasmic reticulum (ER) lumen of non-excitabile cells, where it is considered to be involved mainly in Ca^{2+} storage and buffering. However, there is increasing evidence to implicate the protein in other facets of Ca^{2+} signalling. In this study, we sought to establish more clearly the role of the protein in the regulation of intracellular Ca^{2+} signalling. Generating HeLa cells stably transfected with GFP-tagged calreticulin (GFPCRT) allowed us to select cells by FACS in which calreticulin was expressed at ten times its endogenous levels. Using transiently expressed aequorin as a Ca^{2+} indicator in these cells, we investigated the role of calreticulin in intracellular Ca^{2+} storage, IP_3 -mediated Ca^{2+} release, and capacitative Ca^{2+} entry. The data showed that the capacity of the ionomycin-sensitive Ca^{2+} store was doubled in over-expressing cells, indicating that although calreticulin has a role in Ca^{2+} storage within the lumen, other luminal proteins are also likely to be involved. No difference was observed in the release of Ca^{2+} from the IP_3 -sensitive store in response to prolonged single stimulation with histamine in the absence of extracellular Ca^{2+} , but use of short, sequential pulses of histamine and ATP revealed that calreticulin may exert an effect upon IP_3 -mediated Ca^{2+} release. Two different experimental approaches indicated that calreticulin participates in the regulation of capacitative Ca^{2+} entry. In the presence of extracellular Ca^{2+} , the histamine-generated cytosolic Ca^{2+} signal was significantly lower in GFPCRT cells than those in control cells. Induction of capacitative Ca^{2+} entry by complete emptying of the store using the SERCA pump inhibitor, cyclopiazonic acid also showed that the influx component was significantly reduced in the GFPCRT cells. Use of ER-targeted apoaquorin acting as a luciferase demonstrated that the resting ER free $[\text{Ca}^{2+}]$ in the GFPCRT cells was lower than that in control cells. These data implicate calreticulin in the control of IP_3 -mediated Ca^{2+} release and capacitative Ca^{2+} entry, which may involve direct interaction with Ca^{2+} signalling components or control of ER free $[\text{Ca}^{2+}]$.

INTRODUCTION

Transient increases in cytosolic $[\text{Ca}^{2+}]$ control a myriad of cellular events ranging from fertilization and cellular differentiation to programmed cell death [1,2]. In non-excitabile cells, agonist-dependent rises in cytosolic $[\text{Ca}^{2+}]$ result from the release of Ca^{2+} stored within the endoplasmic reticulum (ER), through stimulation of inositol 1,4,5-trisphosphate (IP_3) receptors, and subsequent influx of Ca^{2+} through the plasma membrane [3,4].

Within the ER, Ca^{2+} is stored by a number of Ca^{2+} sequestering proteins that have low-affinity, high-capacity Ca^{2+} binding sites located within their acidic C-termini. These include the so-called glucose response proteins, grp78/BiP and grp94/endoplasmic reticulum chaperone, as well as calreticulin [5]. Calreticulin is a 46kDa protein with two Ca^{2+} binding regions; a high-affinity ($K_d = 1.6 \mu\text{M}$), low-capacity ($B_{\text{max}} = 1 \text{ mol/mol}$ of protein) site in its central proline-rich P-domain, and a low-affinity ($K_d = 0.3\text{--}2.0 \text{ mM}$), high-capacity ($B_{\text{max}} = 20\text{--}50 \text{ mol/mol}$ of protein) site comprised of aspartic and glutamic acid residues in its C-domain [6]. Indirect evidence, based essentially upon its Ca^{2+} binding characteristics and cellular distribution within the ER, led to the proposal that calreticulin is the major Ca^{2+} storage protein in non-muscle cells [7–9]. This was corroborated by the use of antisense nucleotides to specifically decrease calreticulin

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expression in NG-108-15 neuroblastoma X glioma cells, which resulted in a commensurate drop in the ionomycin-sensitive store in those cells [10].

In addition to its proposed role in storage, a number of observations have suggested that calreticulin might have a more profound role in the control of Ca^{2+} signalling, such as regulation of Ca^{2+} release from the ER, as well as store-operated capacitative Ca^{2+} entry (CCE) across the plasma membrane. Most of these have come from studies in which calreticulin has been over-expressed in a variety of different cell types, which have produced some conflicting results with regard to both IP_3 -mediated Ca^{2+} release and CCE [11–14]. In mouse L fibroblasts stably transfected with a calreticulin expression vector, which resulted in a 60% increase in the amount of calreticulin compared to normal cells, the release of Ca^{2+} in response to ATP was enhanced [13]. In contrast, IP_3 -mediated Ca^{2+} release was unaffected in transiently transfected HeLa cells where calreticulin was over-expressed 3.5 fold than that in non-transfected cells [11]. Similarly, calreticulin over-expression had no effect upon IP_3 -mediated Ca^{2+} release in *Xenopus* oocytes [14], although more recent studies have shown that it inhibits repetitive Ca^{2+} waves induced by IP_3 in these cells [12]. Bastianutto et al. [11] proposed that the depressed CCE they observed arose from incomplete store depletion in over-expressing cells, whereas others have produced data suggesting that calreticulin might actually regulate CCE [13,14].

Whilst these over-expression studies have suggested a more profound role for calreticulin in Ca^{2+} signalling in addition to Ca^{2+} storage, the mechanisms by which it regulates these processes remain to be established. Furthermore, interpretation of the data in studies performed thus far has been complicated by the likely contribution of other Ca^{2+} -binding proteins to these processes, such as *grp78/BiP*, whose over-expression has been shown recently to affect storage capacity in HeLa cells [15].

Therefore, in this study, we have generated stably transfected HeLa cells that express a green fluorescent protein (GFP)-calreticulin fusion protein allowing us to use fluorescence activated cell sorting (FACS) to select cells in which calreticulin is expressed at 10 times its endogenous levels. In these cells, there was a greater contribution of calreticulin to the Ca^{2+} storage capacity of the cell relative to other Ca^{2+} -binding proteins, permitting a clearer assessment of its role in Ca^{2+} storage and signalling. Furthermore, we have utilized ER-targeted apoaquorin acting as a luciferase to address the important question whether calreticulin might affect IP_3 induced Ca^{2+} release and CCE by regulation of ER free $[\text{Ca}^{2+}]$.

MATERIALS AND METHODS

Materials

Enzymes and reagents used in the PCR and preparation and analyses of plasmids were obtained from Promega (Southampton, UK). The S65T GFP variant [16], was obtained from Clontech, Palo Alto, USA. The TA cloning vector pCR3 was from Invitrogen (The Netherlands). Tissue culture reagents came from Sigma (UK) and the G418 from Gibco BRL (UK). The enhanced chemiluminescence system (ECL) was supplied by Amersham (Little Chalfont, Bucks, UK). Coelenterazine was purchased from Molecular Probes (Eugene, OR, USA). The horse-radish peroxidase coupled donkey anti-rabbit antibody was purchased from Jackson Immunoresearch Laboratories (San Francisco, USA). Protein concentration was assessed by the bicinchoninic acid procedure (BCA assay kit, Pierce, UK). All other reagents were of Analar grade and obtained from either Sigma or Fisons (Loughborough, UK).

Methods

Generation of GFPCRT chimeric cDNA

cDNAs containing the whole of the calreticulin protein-coding sequence and the S65T variant of GFP were used in a two stage PCR to generate a cDNA fusion protein comprising: calreticulin ER signal peptide/GFP(S65T)/calreticulin/KDEL (GFPCRT) [17]. The PCR generated products were cloned into pCR3, downstream of the CMV immediate-early promoter.

Production of stable cell lines expressing GFPCRT

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 $\mu\text{g}/\text{ml}$ penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 $\mu\text{g}/\text{ml}$ amphotericin B. Cells were transfected with plasmid DNA using a standard Ca^{2+} phosphate co-precipitation method. Seventy-two hours post transfection cells were selected on G418 (400 $\mu\text{g}/\text{ml}$). The level of expression of the recombinant GFPCRT fusion protein in colonies of G418 resistant cells was initially determined with epi-fluorescence microscopy by visualization of GFP fluorescence emission at 515 nm following excitation at 488 nm. The brightest fluorescent clones (GFPCRT cells) were then selected by FACS (Becton Dickinson FACS 440) (excitation at 488 nm and emission detected through a band pass filter of 535 nm \pm 15 nm).

Western blot analysis

Cells were washed twice in phosphate buffered saline (PBS) (NaCl, 120 mM; KCl, 2.7 mM; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 10 mM, pH 7.4) and harvested in KRH (NaCl, 120 mM;

KCl, 4.8 mM; KH_2PO_4 , 1.2 mM; MgSO_4 , 1.2 mM; CaCl_2 , 1.3 mM; HEPES, 25 mM, pH 7.4 at 37°C), with the following additions: 1 mM EDTA, 0.5% NP40 (v/v), 1 mM PMSF, and 5 mg/ml (each) leupeptin, chymostatin, antipain, aprotinin, all diluted in KRH. The cell suspension was incubated on ice for 30 min and nuclei removed by centrifugation at 900 g for 5 min. Following separation by electrophoresis under reducing conditions through a 10% SDS-polyacrylamide gel, 3 μg cell protein was transferred electrophoretically onto a Hybond-C extra nitrocellulose membrane. The membrane was blocked by incubation in blocking buffer, TBST (150 mM NaCl, 10 mM TrisHCl, pH 7.6 and 0.5% Tween20) containing 5% (w/v) dried skimmed milk, for 1 h at room temperature. The transferred proteins were then probed with either rabbit polyclonal anti-calreticulin [17] or anti-GFP antibody, diluted appropriately in blocking buffer, overnight at 4°C. The membrane was washed extensively in TBST, exposed to a horseradish peroxidase conjugated donkey anti-rabbit IgG antibody for 1 h at room temperature and then washed extensively with TBST. Primary antibody binding was visualized following incubation of the membrane in the peroxidase substrate buffer, supplied with the ECL system.

Intracellular Ca^{2+} measurements

Sub confluent HeLa cells were infected with recombinant replication deficient adenovirus vectors carrying cDNA for apoequorin targeted to the endoplasmic reticulum (ER) (RADER) or a cytosolic firefly-luciferase chimera (RADLA) [18]. The infected cells were detached 24 h later and re-seeded onto glass coverslips. Sodium butyrate (5 mM) was included in the culture medium for 18 h prior to experiments to enhance expression of the recombinant proteins.

For the measurements of cytosolic $[\text{Ca}^{2+}]$, cytosolic recombinant apoequorin was converted to the photoprotein by adding coelenterazine (final concentration 2 μM) at least 4 h before the experiments. The coverslips were inverted over the reservoir of a plastic perfusion chamber which was maintained at 37°C (in a dark box), and brought into contact with a fibre optics bundle attached to a 3-stage image intensified photon counting, charged coupled device (CCD) camera [19]. Cells were perfused for at least 10 min in KRH to remove excess coelenterazine. The fractional discharge of aequorin was calculated from the total light emitted by the photoprotein at the end of each experiment by exposing the cells to water containing 5 mM CaCl_2 to discharge unconsumed aequorin. This value was used to convert light emission into $[\text{Ca}^{2+}]$ [20].

Cells expressing the ER targeted apoequorin were loaded onto the perfusion chamber and perfused with KRH containing 2 μM coelenterazine throughout [18].

All cellular manipulations were delayed for at least 10 min to allow stabilization of light production. As there was no consumption of the photoprotein using this method, calibration of the light signal was calculated as the ratio of light emission to total light emission, assessed by exposing the cells to water containing 5 mM CaCl_2 at the end of each experiment.

Statistical analysis of data

Unless otherwise specifically stated, experiments were carried out in triplicate on at least three separate occasions. All statistical analysis of data was performed using the *t*-test for unpaired samples (InStat, GraphPad Software, San Diego, USA).

RESULTS

Generation of stable cell lines

Following transfection with the plasmid encoding the GFP-calreticulin fusion protein, stable transfectants were selected using G418, and macroscopic colonies with the brightest fluorescence, as assessed by fluorescence microscopy, were expanded. The brightest 10% of these clones (GFPCRT cells) were selected by FACS (Fig. 1). As Figure 1 shows there was a degree of heterogeneity in GFP fluorescence from cells of the same clone, which is in agreement with previous reports describing the stable expression of GFP [21]. In order to avoid any variation that may have arisen owing to this heterogeneity, cells were subjected to FACS analysis every 2–3 weeks in order to select only the brightest cells with similar fluorescence intensities (data not shown).

The populations of cells were selected by their fluorescence intensity on the premise that these clones would be expressing the highest levels of exogenous calreticulin. The actual level of expression of calreticulin in the FACS selected populations was verified by immuno-blot analyses of cell extracts with antibodies to both GFP and calreticulin [17]. Typical immuno-blots of extracts from GFPCRT cells and control HeLa cells are shown in Figure 2. The antibody raised to human calreticulin identified a band of approximately 60 kDa, in agreement with the expected mobility of calreticulin in SDS-PAGE gels, and another at approximately 90 kDa for the GFP-calreticulin fusion protein, again as anticipated. As shown in Panel B, this latter protein was also recognized by antiserum raised to GFP. Densitometric analysis showed that the intensity of this band with the anti-calreticulin antibody was 10 times that of the endogenous calreticulin band at 60 kDa. Thus, the level of expression of the recombinant GFP-calreticulin in the GFPCRT cells was 10 times that of the endogenous protein.

Having established GFPCRT cells that expressed calreticulin at ten times its normal levels, we proceeded

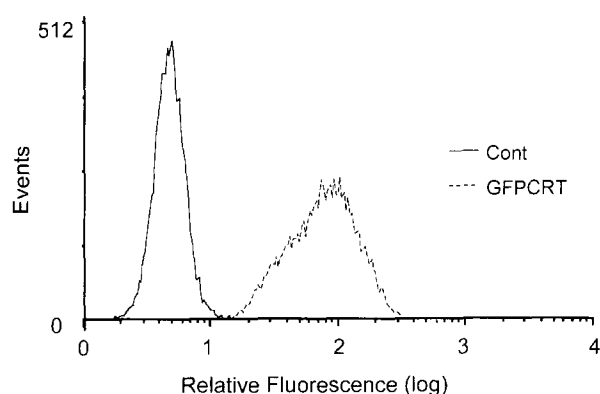


Fig. 1 Relative distribution of fluorescence in GFPcrt cells compared to control cells. Cells were detached from their substrate, resuspended at $1 \times 10^6/\text{ml}$ analysed by FACS (excitation 488 nm; emission 535 ± 15 nm). The distribution of fluorescence in the two populations of cells is shown plotted as the number of events (cells) against relative fluorescence. There was a degree of variation in the fluorescence of the GFPcrt cells and so only the brightest 10% of the population was selected. Cells were subjected to such analysis every 2–3 weeks to avoid variation owing to different levels of expression of the GFPcrt fusion protein.

to investigate the roles of the protein in intracellular Ca^{2+} homeostasis by comparing intracellular Ca^{2+} signals in the GFPcrt cells to those in control HeLa cells. By transiently expressing the Ca^{2+} sensitive photoprotein aequorin, appropriately targeted to the relevant intracellular location, we compared the size of the ionomycin-sensitive Ca^{2+} pools in the two populations of cells, the effect of calreticulin over-expression upon agonist-induced IP_3 -mediated Ca^{2+} release and capacitative Ca^{2+} entry (CCE), as well as the ER free $[\text{Ca}^{2+}]$.

Calreticulin over-expression increases the size of the ionomycin sensitive Ca^{2+} pool

We first investigated whether the GFPcrt cells differed from control cells with respect to their Ca^{2+} storage capacity. Light emission from populations of GFPcrt cells and control HeLa cells expressing the luciferase-aequorin chimera, which locates exclusively in the cytosol [22], was monitored and the fractional luminescence used to calculate cytosolic $[\text{Ca}^{2+}]$. Experiments were performed in triplicate on three separate occasions. Figure 3 shows the effect of $2 \mu\text{M}$ ionomycin on cytosolic $[\text{Ca}^{2+}]$ in both control and GFPcrt cells. In the absence of extracellular Ca^{2+} , the basal cytosolic $[\text{Ca}^{2+}]$ reported by appropriately located aequorin was similar in both populations of cells, i.e. approximately $0.1 \mu\text{M}$. Addition of ionomycin increased cytosolic $[\text{Ca}^{2+}]$ to $0.60 \pm 0.09 \mu\text{M}$ in GFPcrt cells. However, similar treatment of control cells elicited a cytosolic Ca^{2+} transient that was substantially lower, reaching a peak value of only $0.36 \pm 0.03 \mu\text{M}$. Integrating the area under the curve (AUC) to give an

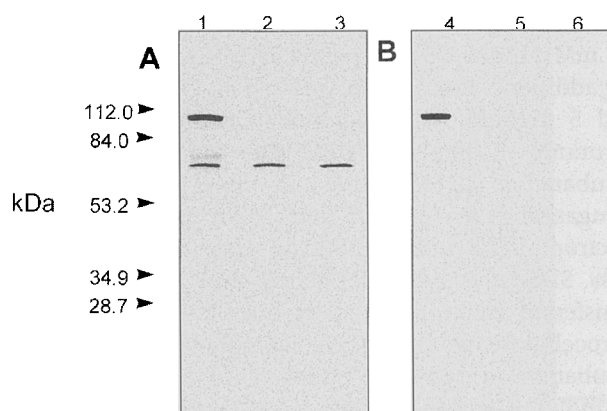


Fig. 2 Western blot analysis of cell extracts probed with polyclonal rabbit anti-calreticulin and anti-GFP antibody. Crude cell extracts were prepared as described in the Methods sections. Three micrograms of cell protein was subjected to SDS/PAGE, transferred to a nitrocellulose membrane and probed with rabbit polyclonal anti-calreticulin or anti-GFP. Antibody binding was visualized with goat anti-rabbit IgG conjugated to horseradish peroxidase, as described in the Methods section. Panel A: Western blot probed with anti-calreticulin antibody. Lane 1: GFPcrt cell extract shows a band at approximately 60 kDa (endogenous calreticulin) and one at 90 kDa (recombinant calreticulin-GFP fusion protein) with approximately 10 times the intensity of the 60 kDa band. Negatively sorted, non-expressing cells (lane 2) and non-transfected cells (lane 3), show the presence of endogenous calreticulin at approximately 60 kDa, but no positive staining at 90 kDa. Panel B: Western blot probed with anti GFP antibody. Lane 4: GFPcrt cell extract shows a band at 90 kDa, which is identical to that probed with anti-calreticulin antibody. Lanes 2 and 3 correspond to negatively sorted, non-expressing cells and non-transfected cells, respectively.

estimate of the total Ca^{2+} released by this agent, showed that it was significantly higher in GFPcrt cells ($19.05 \pm 2.86 \mu\text{M} \cdot \text{sec}$) than in control cells ($10.27 \pm 1.78 \mu\text{M} \cdot \text{sec}$) ($P < 0.001$). This effect was specifically caused by calreticulin since no changes in the size of the ionomycin-sensitive store were seen in cells in which GFP alone was targeted to the ER [17]. This was as expected since GFP is not a Ca^{2+} -binding protein and has been used in combination with calmodulin to monitor changes in intracellular $[\text{Ca}^{2+}]$, where any effect of GFP *per se* upon Ca^{2+} homeostasis would be problematical [23,24]. Thus, over-expression of calreticulin increases the size of the ionomycin-sensitive pool.

Calreticulin over-expression can alter IP_3 mediated Ca^{2+} release

It has been established that ionomycin releases not only the IP_3 sensitive intracellular Ca^{2+} stores, but also the non-acidic stores of Ca^{2+} into the cytosol [25]. In order to investigate whether over-expression of calreticulin specifically affected the release of Ca^{2+} from IP_3 sensitive stores, we compared cytosolic Ca^{2+} signals in GFPcrt and control cells elicited by agonists that mobilise intracellular Ca^{2+} following the generation of IP_3 [26,27].

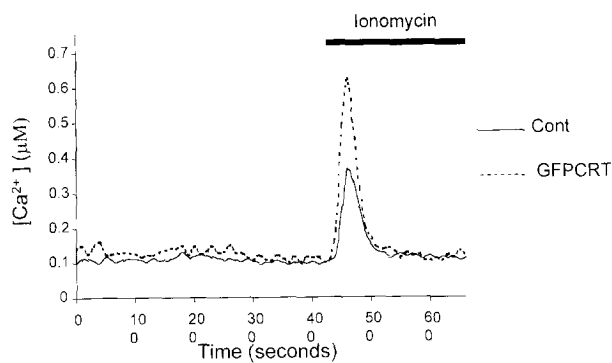


Fig. 3 Cytosolic Ca^{2+} transients in GFPCRT and control cells induced by ionomycin. GFPCRT and control HeLa cells expressing recombinant cytosolic luciferase-aequorin were cultured on glass coverslips and perfused with KRH containing 1 mM EGTA. Light production was monitored through a fibre optic probe attached to a photon counting camera. The Ca^{2+} ionophore ionomycin (2 μM) was then included in the perfusion medium, as indicated. Free Ca^{2+} values were derived from luminescence counts obtained from the cells as described in the Methods section. The data shown are the mean results of nine experiments for each cell type.

Initially, experiments were carried out exposing the cells to a single prolonged challenge with histamine (100 μM) over 200 s in the absence of extracellular Ca^{2+} (Fig. 4). As with ionomycin previously, these experiments were carried out on three occasions in triplicate. The mean peak amplitude of the histamine evoked transient in GFPCRT cells was $0.32 \pm 0.003 \mu\text{M}$ compared to $0.34 \pm 0.07 \mu\text{M}$ in control cells. Similarly, there was no significant difference in the total Ca^{2+} released as estimated by calculating the AUCs between control cells ($9.33 \pm 1.68 \mu\text{M} \cdot \text{s}$) and over-expressing cells ($9.61 \pm 1.49 \mu\text{M} \cdot \text{s}$) ($P > 0.5$).

Although these data suggested that there was no difference in the size of the IP_3 sensitive store between calreticulin overexpressing cells and control cells, we next used an established protocol in which consecutive short challenges with agonist are made in the absence of extracellular Ca^{2+} to determine more precisely the size of the IP_3 sensitive Ca^{2+} store [11]. In this protocol, subtle changes in the size of the IP_3 sensitive Ca^{2+} store have been revealed, avoiding the problems of receptor desensitization arising from prolonged exposure to agonist or influx of Ca^{2+} through the plasma membrane. The data from these experiments are shown as an average trace of all the experiments ($n=16$) in Figure 5 with the data for peak height and total Ca^{2+} released given in Table 1. In contrast to the single prolonged challenge with histamine, differences were apparent between the control cells and GFPCRT cells upon these shorter consecutive applications of agonists. As these data show, a short 30 s pulse of 100 μM histamine applied to the control cells in the absence of extracellular Ca^{2+} caused a rapid and transient rise in cytosolic $[\text{Ca}^{2+}]$ which was significantly larger, both in terms of maximal cytosolic $[\text{Ca}^{2+}]$ and total

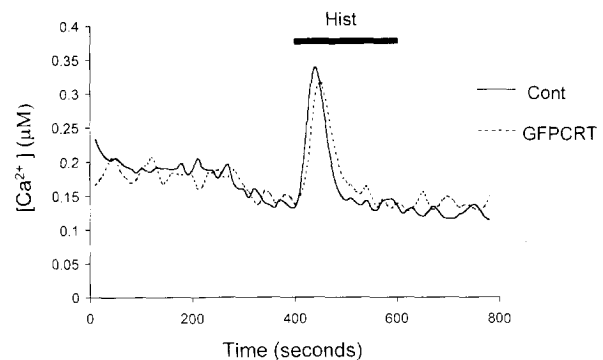


Fig. 4 Cytosolic Ca^{2+} transients in GFPCRT and control cells induced by prolonged single stimulation with histamine. Cells (see legend to Figure 3) expressing cytosolic aequorin were perfused in KRH containing 1 mM EGTA. A 30 s challenge was made with histamine (100 μM). Free Ca^{2+} values were derived from luminescence counts obtained from the cells as described in the Methods section. The data shown are the mean results of nine experiments for each cell type.

Ca^{2+} released (as estimated by the AUCs, calculated with corrections for the differences in baseline cytosolic $[\text{Ca}^{2+}]$) than that in GFPCRT cells ($P < 0.0001$). Following washout of the histamine, a 30 s pulse of 100 μM ATP, selected to avoid receptor desensitization, was applied to the cells. The ATP-induced transient, both in terms of peak cytosolic Ca^{2+} and estimated total Ca^{2+} released, was significantly reduced in the control cells compared to their response to histamine ($P < 0.0001$). However, in the GFPCRT cells, the Ca^{2+} transient that resulted from ATP treatment was very similar to initial response elicited by histamine, and was significantly larger than the response

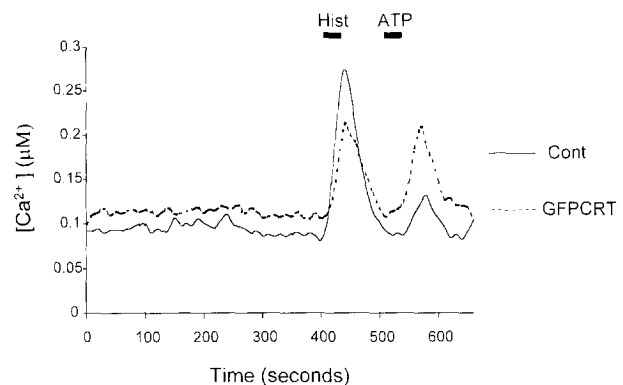


Fig. 5 Cytosolic Ca^{2+} transients in GFPCRT and control cells induced by short, successive stimulations with histamine and ATP. Cells (see legend to Fig. 3) expressing cytosolic aequorin were perfused in KRH containing 1 mM EGTA. A 30 s challenge was first made with histamine (100 μM). After washout of this agonist a second challenge was made with ATP (100 μM), again for 30 s. Successive stimulation with different agonists avoids receptor desensitisation resulting from prolonged exposure to the same agonist. Free Ca^{2+} values were derived from luminescence counts obtained from the cells as described in the Methods section. The data shown are the mean results of 16 experiments for each cell type.

Table 1 Estimations of the total Ca^{2+} released upon sequential stimulation with histamine and ATP. The data shown are integrals of the areas under the curve (AUCs), calculated using the trapezoidal rule, using the data shown in Figure 5 ($n = 16$). As indicated in the text, the AUCs were calculated taking into account the differences in the baseline cytosolic Ca^{2+}

	Peak amplitude (μM) (\pm s.d.)	Area under the curve ($\mu\text{M}\cdot\text{s}$) (\pm s.d.)
Control		
+ histamine	0.27 ± 0.04	8.52 ± 0.96
+ ATP	0.13 ± 0.02	1.08 ± 0.23
GFPCRT		
+ histamine	0.21 ± 0.02	5.42 ± 0.54
+ ATP	0.21 ± 0.02	4.05 ± 0.69

in control cells ($P < 0.0001$). In all experiments conducted, the release in response to the second challenge was always much bigger in the GFPCRT cells than control cells. These results suggest that a portion of the IP_3 sensitive Ca^{2+} pool remains intact after the initial agonist challenge in GFPCRT cells, whereas in control cells the initial challenge clearly depletes most, if not all, of the Ca^{2+} in the same pool. By summing the AUCs to estimate the total Ca^{2+} released by both agonists, there was no significant difference between control cells and overexpressers ($P > 0.5$).

Calreticulin over-expression depresses capacitative Ca^{2+} entry

Cytosolic Ca^{2+} signals in HeLa cells elicited by agonists applied in the presence of extracellular Ca^{2+} are biphasic and arise from a combination of release of Ca^{2+} from intracellular IP_3 sensitive Ca^{2+} stores and CCE through the plasma membrane [28]. In order to ascertain whether calreticulin affected CCE, we measured the contribution made to the Ca^{2+} transient by each of these components using two different experimental approaches. Application of $100 \mu\text{M}$ histamine in the presence of extracellular Ca^{2+} evoked an immediate biphasic transient rise in cytosolic free $[\text{Ca}^{2+}]$, monitored by the recombinant cytosolic aequorin, in both populations of cells (Fig. 6). The peak amplitude of the cytosolic Ca^{2+} transient in Ca^{2+} signal in GFPCRT cells ($0.31 \pm 0.02 \mu\text{M}$) was significantly reduced compared to that in control cells ($0.39 \pm 0.04 \mu\text{M}$) ($P < 0.0001$). Similarly, the total Ca^{2+} released was significantly smaller ($27.53 \pm 5.06 \mu\text{M}\cdot\text{s}$) than in GFPCRT cells ($49.34 \pm 5.31 \mu\text{M}\cdot\text{s}$) ($P < 0.0001$). The kinetics of the agonist-induced Ca^{2+} transients were also different in the two cell types. In control cells, the rate at which the cytosolic Ca^{2+} transient reached its maximum value was $0.0049 \mu\text{M}/\text{s}$, whereas in GFPCRT cells this value was lower at $0.0032 \mu\text{M}/\text{s}$. In contrast, the rate at which the cytosolic Ca^{2+} transients

returned to pre-stimulation levels was faster in GFPCRT cells ($0.0013 \mu\text{M}/\text{s}$) compared to the control population ($0.0010 \mu\text{M}/\text{s}$).

CCE can also be elicited by agents that inhibit sequestration of Ca^{2+} into the ER by SERCA pumps [3,4]. The SERCA pump inhibitor cyclopiazonic acid (CPA) [29] was applied to the cells in the absence of extracellular Ca^{2+} to deplete the ER Ca^{2+} store and activate CCE (Fig. 7). To ensure that the store was completely emptied by this treatment with CPA, both populations of cells were subsequently exposed to $100 \mu\text{M}$ histamine, which revealed no further mobilization of intracellular Ca^{2+} (data not shown). Following washout of CPA, 1.3 mM CaCl_2 was added to the medium bathing these cells, inducing rapid and transient increases in cytosolic $[\text{Ca}^{2+}]$ in both populations of cells (Fig. 7). In control cells, this transient influx caused an increase in cytosolic $[\text{Ca}^{2+}]$ from a resting level of approximately $0.1 \mu\text{M}$ to $0.43 \pm 0.06 \mu\text{M}$ (AUC; $12.86 \pm 0.97 \mu\text{M}\cdot\text{s}$). In contrast, this

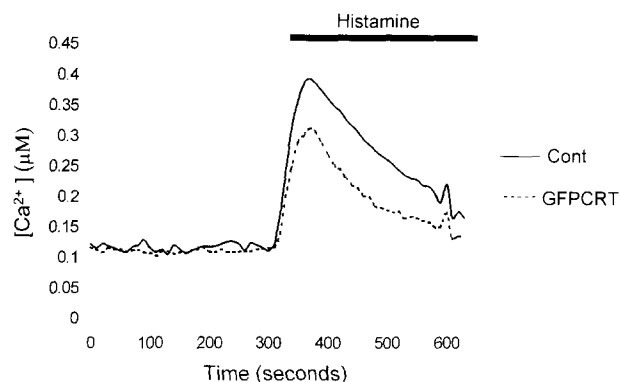


Fig. 6 Histamine-induced cytosolic Ca^{2+} transients in GFPCRT and control cells in the presence of extracellular Ca^{2+} . Cells expressing cytosolic aequorin (see legend to Fig. 3) were perfused in KRH containing 1.3 mM CaCl_2 and exposed to histamine ($100 \mu\text{M}$) for the period indicated by the bar. Free Ca^{2+} values were derived from luminescence counts obtained from the cells as described in the Methods section. The data shown are the mean results of nine experiments for each cell type.

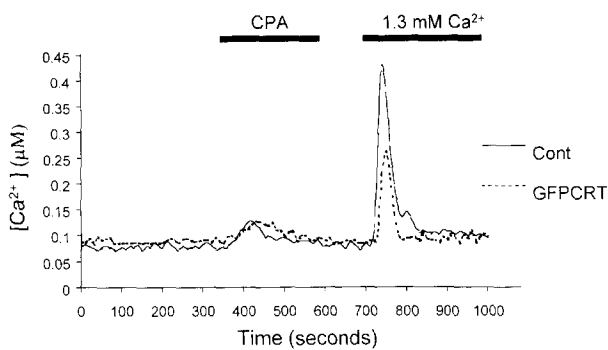


Fig. 7 Cytosolic Ca^{2+} transients in GFPCRT and control cells arising from store operated capacitative Ca^{2+} entry. Cells expressing cytosolic aequorin (see legend to Fig. 3) were perfused in Ca^{2+} free KRH containing 1 mM EGTA. At the time indicated by the bar, the SERCA pump inhibitor cyclopiazonic acid (CPA) (10 μM) was added to the medium bathing the cells. Inhibition of the SERCA pump effectively depletes the IP_3 sensitive Ca^{2+} store. Following washout of the CPA, 1.3 mM Ca^{2+} was restored to the KRH, triggering store operated influx, and the concomitant rise in cytosolic $[\text{Ca}^{2+}]$. Free Ca^{2+} values were derived from luminescence counts obtained from the cells as described in the Methods section. The data shown are the mean results of nine experiments for each cell type.

transient increase was substantially reduced in GFPCRT cells and reached a maximum value of only $0.26 \pm 0.06 \mu\text{M}$ (AUC; $5.35 \pm 0.59 \text{ mM.s}$) ($P < 0.0001$). Thus, these data showed that over-expression of calreticulin depressed CCE.

Both the size of the cytosolic Ca^{2+} transient, resulting from mobilization of intracellular Ca^{2+} , and the influx of Ca^{2+} through the plasma membrane are reported to be controlled by the free $[\text{Ca}^{2+}]$ in the ER [1]. Indirect evidence, based on the Ca^{2+} binding characteristics and cellular distribution, suggests that one of the major functions of calreticulin is to buffer the ER free $[\text{Ca}^{2+}]$ [9]. Thus, in order to assess whether the depression of CCE by calreticulin might be due to an effect upon ER free $[\text{Ca}^{2+}]$, we used ER targeted apoaequorin as a pseudo luciferase [18] to compare directly the resting free $[\text{Ca}^{2+}]$ in the ER of GFPCRT and control HeLa cells. When cells expressing ER targeted apoaequorin were perfused with coelenterazine there was an immediate increase, followed by a slower consistent increase in light production. After a perfusion period of 1000 s, the cells were perfused in water containing 5 mM CaCl_2 and 2 μM coelenterazine in order to expose total apoaequorin expressed by each of the cell populations to saturating $[\text{Ca}^{2+}]$ and coelenterazine [18]. This perfusion resulted in a further rapid increase, followed by a slower increase in light production at an elevated level. The mean value of the elevated level of light production was used as an indicator of the total apoaequorin in the cells, and was used to establish a ratio of light emission in resting cells to total light production, reflecting the free $[\text{Ca}^{2+}]$ in the

ER of both populations of cells. Using this approach, the ratio of the total light emission to light emission at rest was 6.2 ± 1.5 in control cells compared to 11 ± 4 in GFPCRT cells, indicating that the ER resting free $[\text{Ca}^{2+}]$ in the control cells was higher than in GFPCRT cells (Fig. 8).

DISCUSSION

In this study, in order to establish more precisely the role of calreticulin in regulating intracellular Ca^{2+} storage and signalling, we have generated stable HeLa cell lines that hyper-express it. This was achieved by the construction of a fusion protein between calreticulin and GFP, which allowed cells expressing the highest levels of calreticulin to be selected by FACS, utilizing the inherent fluorescence of the GFP. Immunoblot analysis revealed that those cells selected with the highest fluorescent signal expressed calreticulin at levels 10-fold that of the endogenous protein. Despite this, the localization of the exogenous protein was identical to that of the endogenous protein as shown by both immunocytochemistry using an anti-calreticulin antibody, and by GFP fluorescence of live cells (data not shown). Such a finding is in keeping with our previous observations where we have expressed GFPCRT fusion proteins in cells [17] and those of others where calreticulin was over-expressed up to 100 fold in individual cells [30].

The size of the ionomycin-sensitive intracellular Ca^{2+} pool was approximately doubled in GFPCRT cells, both in terms of the peak cytosolic Ca^{2+} signal and the total amount of Ca^{2+} released. Thus, the Ca^{2+} storage capacity is enhanced by increasing intra-lumenal levels of calreti-

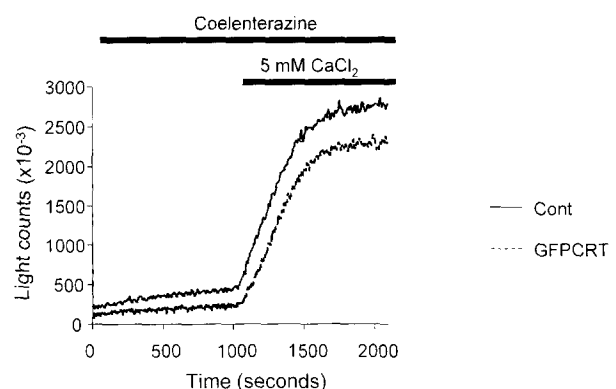


Fig. 8 Comparison of resting ER free $[\text{Ca}^{2+}]$ in GFPCRT and control cells. Cells expressing ER targeted apoaequorin were perfused in KRH. At time 0, 2 μM coelenterazine was added to the perfusion medium and light output was continually monitored. After 1100 s, water containing 5 mM CaCl_2 and 2 μM coelenterazine was added to the cells in order to expose total apoaequorin to saturating $[\text{Ca}^{2+}]$ in excess coelenterazine [17]. The data plotted are the mean values obtained from 9 experiments. Ratios or resting to total light output were obtained from the mean value of light generation between 0 and 1100 (resting) and 1500 and 2000 (total) s.

culin, strongly suggesting that the protein acts as a major Ca^{2+} storage protein within cells. This finding is in line with other studies where intracellular levels of calreticulin have been increased. Bastianutto et al. [11] showed that the capacity of the Ca^{2+} store releasable by another Ca^{2+} ionophore, A23187 was increased two-fold in HeLa cells in which calreticulin levels were elevated 3.5 fold, whilst Mery et al. [13] demonstrated that the Ca^{2+} storage capacity was similarly increased in mouse L-fibroblasts. Interestingly, in the latter study, the calreticulin expression was enhanced only 1.6 fold, yet produced a 2.1 fold increase in stored Ca^{2+} , suggesting perhaps that calreticulin is able to increase Ca^{2+} storage by a mechanism that does not simply involve Ca^{2+} buffering. In our studies, we expressed the protein at 10 times the level of endogenous protein, which still only gave an approximate doubling in Ca^{2+} storage capacity, and these data, taken together with that of Bastianutto et al. [11], indicate that calreticulin is not the only protein within the ER that is responsible for storing Ca^{2+} . There are other Ca^{2+} binding proteins located within the lumen of the ER and, recently, it has been shown that one of these, grp78/BiP, is responsible for storing about 25% of the Ca^{2+} in HeLa cells [15].

Despite the increased Ca^{2+} storage capacity observed in the calreticulin overexpressing cells, as indicated by the ionomycin data, our experiments did not demonstrate that the overall size of the IP_3 -sensitive store was significantly larger in these cells, as the total Ca^{2+} released, by either prolonged single stimulation with histamine, or short sequential stimulations with histamine and then ATP, was unaffected. Perhaps the simplest interpretation of these results is that calreticulin overexpression specifically increases the size of the ionomycin-sensitive store. A recent report showed that there was no effect upon the IP_3 or thapsigargin sensitive store in mouse embryonic stem cells in which calreticulin had been knocked out [31], which may fit with our data here, but previous studies have indicated that the capacity of the IP_3 sensitive store is increased in calreticulin overexpressing cells [11,13]. Our data do not exclude this possibility, which might have been revealed by further agonist stimulation. Bastianutto and co-workers failed to show a difference in cytosolic Ca^{2+} elevations between control cells and calreticulin overexpressing cells upon prolonged single application of agonist suggesting that the release of Ca^{2+} under such conditions may be largely independent of the storage capacity of the cells [11].

It is clear that calreticulin overexpression does exert an effect on the IP_3 -sensitive store, since the sequential double agonist challenge experiments demonstrated altered characteristics of Ca^{2+} release in the GFPCRT cells compared to control cells. Like Bastianutto et al. [11], who first adopted the approach of using short sequential

pulses of agonist to reveal differences in the store, our results showed that the second stimulation released more Ca^{2+} in the calreticulin overexpressing cells. However, in contrast to their observations, our data indicated that the initial agonist response was significantly reduced in these cells. The most straightforward explanation for the enhanced second response in our data is that the reduced initial response did not deplete the IP_3 -sensitive Ca^{2+} store in the GFPCRT cells, such that more could be released upon subsequent stimulation. This raises the question of how calreticulin overexpression causes a diminished initial response. Amongst the possibilities is an increase in SERCA pump activity, such that Ca^{2+} is returned more rapidly to the store following the brief initial stimulation, or, tighter regulation of Ca^{2+} release via the IP_3 receptor. These could result either from direct interaction between calreticulin and the pump or receptor, or, indirectly via an effect on the free lumenal $[\text{Ca}^{2+}]$, which was reduced in our overexpressing cells. There is evidence that indicates that calreticulin is able to affect the activity of SERCA pumps, although these have shown that the effect of calreticulin overexpression is to inhibit repetitive Ca^{2+} waves, rather than an increase in pump activity [12,32].

The final experiments in this study examined the effect of calreticulin over-expression upon CCE. Cytosolic Ca^{2+} signals in HeLa cells elicited by agonists linked to the generation of IP_3 arise from a combination of release of Ca^{2+} from intracellular store and influx of the ion through the plasma membrane. We used two experimental approaches to ascertain whether the levels of expression of calreticulin affected cytosolic Ca^{2+} signals generated in the presence of extracellular Ca^{2+} . In the first of these approaches, cells were challenged with histamine for the duration of the experiment. This treatment gave rise to cytosolic Ca^{2+} signals that were clearly different to those seen in the absence of extracellular Ca^{2+} , reflecting the contribution made by CCE in addition to the initial release from the store. As we had already established that the size of the cytosolic Ca^{2+} transient in response to prolonged stimulation with histamine in GFPCRT cells was equivalent to that in control cells, the difference in signals observed in the presence of extracellular Ca^{2+} could have arisen only as a result of a diminished CCE component in cells that over-expressed calreticulin. Furthermore, in GFPCRT cells, the rate at which the maximal signal was attained was slower than in control cells, whereas, in contrast, the rate at which cytosolic $[\text{Ca}^{2+}]$ returned to pre-stimulation levels was faster in the GFPCRT population. Thus, these data raised the possibility that calreticulin exerts some control over CCE. This was confirmed subsequently when CCE was activated by first treating cells with CPA in the absence of extracellular Ca^{2+} , before restoring extracellular Ca^{2+} once the store had been emptied.

Diminished CCE has previously been observed in calreticulin over-expressing cells. Mery et al. [13] demonstrated it in mouse L-fibroblasts by assessing Mn^{2+} influx and subsequent quenching of Fura2 fluorescence. Bastianutto et al. [11], using a similar approach to that described here, proposed that the diminished influx component activated by agonist in their studies arose from proportionally less emptying of the store in over-expressing cells, thereby subsequently generating a weaker signal to activate the influx. However, in our experiments, chronic treatment of both populations of cells with CPA completely emptied the IP_3 sensitive store, as subsequent treatment with histamine revealed no further mobilization of intracellular Ca^{2+} . Since CPA is a reversible inhibitor, then a more active pump in the overexpressing cells might account for the decreased influx owing to more rapid replenishment of the store from the cytosol in those cells. However, as stated, there is no evidence to indicate such an effect on pump activity by calreticulin, whilst crucially, we also observed the same effect upon influx in GFP-CRT cells when the dead-end SERCA inhibitor, thapsigargin was used (data not shown). Thus, it appears that calreticulin within the lumen is able to control CCE. Appropriately targeted apoaequorin acting as a luciferase to monitor the ER free $[\text{Ca}^{2+}]$ in both control and over-expressing cells [17] showed that the ER free $[\text{Ca}^{2+}]$ was lower in the GFP-CRT cells than in control cells. It now remains to determine whether this lowering of the ER free $[\text{Ca}^{2+}]$ contributes to the reduced CCE, or, if there is a direct involvement of calreticulin in controlling CCE that is independent of the effect upon the ER free $[\text{Ca}^{2+}]$. It has been proposed that CCE occurs through a putative plasma membrane channel whose activity is controlled either by a soluble factor or by physical interaction with components of the ER [3]. The simplest mechanism that can be envisaged is a direct interaction between calreticulin and the plasma membrane Ca^{2+} channel itself. Calreticulin has been reported to interact with other ER luminal proteins such as PDI [33], as well as the ER membrane SERCA 2 pump by which it controls the physical characteristics of cytosolic Ca^{2+} signals [32]. Characterization of the pathway that triggers CCE, together with the identity of the channel itself, should help in elucidating the mechanism by which calreticulin regulates CCE.

Finally, we have shown previously that calreticulin expression is induced by stress [34,35]. The data presented in this present study suggest that changes in expression may have profound effects upon intracellular Ca^{2+} storage and signalling, making it imperative to investigate further the regulation of calreticulin expression.

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REFERENCES

- Berridge MJ. Inositol trisphosphate and calcium signalling. *Nature* 1993; **361**: 315–325.
- Clapham DE. Calcium signaling. *Cell* 1995; **80**: 259–268.
- Berridge MJ. Capacitative calcium entry. *Biochem J* 1995; **312**: 1–11.
- Putney JW. Type 3 inositol 1,4,5-trisphosphate receptor and capacitative calcium entry. *Cell Calcium* 1997; **21**: 257–261.
- Macer DRJ, Koch GLE. Identification of a set of calcium-binding proteins in reticuloplasm, the luminal content of the endoplasmic reticulum. *J Cell Sci* 1988; **91**: 61–70.
- Baksh S, Michalak M. Expression of calreticulin in *Escherichia coli* and identification of its Ca^{2+} binding domains. *J Biol Chem* 1991; **266**: 21458–21456.
- Milner RE, Baksh S, Shemanko C et al. Calreticulin, and not calsequestrin, is the major calcium-binding protein of smooth muscle sarcoplasmic reticulum and liver endoplasmic reticulum. *J Biol Chem* 1991; **266**: 7155–7156.
- Lytton J, Nigam SK. Intracellular Ca^{2+} : molecules and pools. *Curr Opin Cell Biol* 1992; **4**: 220–224.
- Michalak M, Milner RE, Burns K, Opas M. Calreticulin. *Biochem J* 1992; **285**: 681–692.
- Liu NG, Fine RE, Simons E, Johnson RJ. Decreasing calreticulin expression lowers the Ca^{2+} response to bradykinin and increases sensitivity to ionomycin in NG-108-15 cells. *J Biol Chem* 1994; **269**, **46**: 28635–28639.
- Bastianutto C, Clementi E, Codazzi F et al. Over-expression of calreticulin increases the Ca^{2+} capacity of rapidly exchanging Ca^{2+} stores and reveals aspects of their luminal microenvironment and function. *J Cell Biol* 1995; **130**: 847–855.
- Camacho P, Lechleiter JD. Calreticulin inhibits repetitive intracellular Ca^{2+} waves. *Cell* 1995; **82**: 765–771.
- Mery L, Mesaeli N, Michalak M, Opas M, Lew DP, Krause KH. Over-expression of calreticulin increases intracellular Ca^{2+} storage and decreases store-operated Ca^{2+} influx. *J Biol Chem* 1996; **271**: 9332–9339.
- Delisle S, Longo FJ, Wintermantel MR, Welsh MJ, Clark RA. Calreticulin over-expression in *Xenopus* oocytes - effect of the inositol(3)-induced Ca^{2+} signal. *Clinical Research* 1994; **42**: A 354.
- Lievremont JP, Rizzuto R, Hendershot L, Meldolesi J. BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca^{2+} . *J Biol Chem* 1997; **272**: 30873–30879.
- Heim R, Cubitt AB, Tsien RY. Improved green fluorescence. *Nature* 1995; **373**: 663–664.
- Roderick HL, Campbell AK, Llewellyn DH. Nuclear localisation of calreticulin in vivo is enhanced by its interaction with glucocorticoid receptors. *FEBS Letters* 1997; **405**: 181–185.
- Kendall JM, Badminton MN, Sala-Newby GB, Campbell AK, Rembold CM. Recombinant apoaequorin acting as a pseudo-luciferase reports micromolar changes in the endoplasmic reticulum free Ca^{2+} of intact cells. *Biochem J* 1996; **318**: 383–387.
- Campbell AK, Trewavas AJ, Knight MR. Calcium imaging shows

- differential sensitivity to cooling and communication in luminous transgenic plants. *Cell Calcium* 1996; **19**: 211–218.
20. Cobbold PH, Lee JAC. Aequorin measurements of cytoplasmic free calcium. In: McCormack JG, Cobbold PH. (eds) *Cellular calcium: a practical approach*. New York. Oxford University Press, 1991; pp. 55–80
 21. Cheng LZ, Fu J, Tsukamoto A, Hawley RG. Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. *Nat Biotechnol* 1996; **14**: 606–609.
 22. Badminton MN, Campbell AK, Rembold CM. Differential regulation of nuclear and cytosolic Ca^{2+} in HeLa cells. *J Biol Chem* 1996; **271**: 31210–31214.
 23. Persechini A, Lynch JA, Romoser VA. Novel fluorescent indicator proteins for monitoring free intracellular Ca^{2+} . *Cell Calcium* 1997; **22**: 209–216.
 24. Miyawaki A, Llopis J, Heim R et al. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* 1997; **388**: 882–887
 25. Fasolato C, Zottini M, Clementi E, Zacchetti D, Meldolesi J, Pozzan T. Intracellular Ca^{2+} pools in PC12 cells - 3 intracellular pools are distinguished by their turnover and mechanisms of Ca^{2+} accumulation, storage, and release. *J Biol Chem* 1991; **266**: 20159–20167.
 26. Smit MJ, Bloemers SM, Leurs R et al. Short term desensitization of the histamine-H1 receptor in human HeLa cells - involvement of protein kinase C dependent and independent pathways. *British Journal of Pharmacology* 1992; **107**: 448–455.
 27. Smit MJ, Leurs R, Bloemers SM et al. Extracellular ATP elevates cytoplasmic free Ca^{2+} in hela cells by the interaction with a 5'-nucleotide receptor. *Eur J of Pharmacol* 1993; **247**: 223–226.
 28. Bootman MD, Berridge MJ, Taylor CW. All-or-nothing Ca^{2+} mobilization from the intracellular stores of single histamine-stimulated HeLa Cells. *J Physiol Lon* 1992; **450**: 163–178.
 29. Seidler NW, Jona I, Vegh M, Martonosi A. Cyclopiazonic acid is a specific inhibitor of the Ca^{2+} -ATPase of sarcoplasmic reticulum. *J Biol Chem* 1989; **264**: 17816–17823.
 30. Sonnichsen B, Fullekrug J, Van PN, Diekmann W, Robinson DG, Mieskes G. Retention and retrieval - both mechanisms co-operate to maintain calreticulin in the endoplasmic reticulum. *J Cell Sci* 1994; **107**: 2705–2717.
 31. Coppelino MG, Woodside MJ, Demareux N, Grinstein S, StArnaud R, Dedhar S. Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. *Nature* 1997; **386**: 843–847.
 32. John LM, Lechleiter JD, Camacho P. Calreticulin exhibits SERCA 2 isoform specificity in the modulation of Ca^{2+} waves in *Xenopus* oocytes. *Biophys J* 1997; **72**: PWP265
 33. Baksh S, Burns K, Andrin C, Michalak M. Interaction of calreticulin with protein disulphide isomerase. *J Biol Chem* 1995; **270**: 31338–31344
 34. Llewellyn DH, Kendall JM, Sheikh FN, Campbell AK. Induction of calreticulin expression in HeLa cells by depletion of the endoplasmic reticulum Ca^{2+} store and inhibition of N-linked glycosylation. *Biochem J* 1996; **318**: 550–555
 35. Llewellyn DH, Roderick HL, Rose S. KDEL receptor expression is not coordinately up-regulated with ER stress-induced reticuloplasm expression in HeLa cells. *Biochem Biophys Res Commun* 1997; **240**: 36–40